

Negative effects of vertebrate on invertebrate herbivores mediated by enhanced plant nitrogen content

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Running Head: Trait-mediated indirect interactions

**Trait-mediated indirect effects of vertebrate herbivores on invertebrate herbivores:
enhanced plant quality negatively affects grasshoppers**

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24 ***Abstract***

25 Classic theory holds that the main interaction within the herbivore guild is competition,
26 based on research focused on co-occurring, similarly-sized species that reduce the quantity of
27 shared plant resources. However, a growing body of research is increasingly appreciating the
28 role of herbivorous species as initiators of indirect interactions. Indirect effects may be as
29 important as competition, especially among differently-sized herbivores, given that small
30 terrestrial herbivores appear to be more sensitive to alterations of plant quality than plant
31 quantity. In this study, we first tested in the field whether large vertebrate herbivores (cattle
32 *Bos taurus*) exerted a negative effect on smaller insect herbivores (grasshopper
33 *Euchorthippus unicolor*) through their overlapping foraging preferences for the dominant
34 grass *Leymus chinensis*. We then assessed with field and laboratory experiments if the
35 negative effect was a result of competition for resources (i.e. plant quantity) or an indirect
36 effect mediated by plant quality. Our results showed that grazing by large herbivores caused a
37 significant decline in grasshopper population density and individual performance (survival,
38 size and weight of both female and male *E.unicolor*). Interestingly, the negative effects of
39 large herbivores on grasshoppers were not mediated by reduced resource availability, but by
40 enhanced quality (nitrogen content) of plant resources. The biomass of shared dominant grass
41 *L. chinensis* was marginally affected by large herbivore grazing, whereas N content in *L.*
42 *chinensis* was significantly increased in grazed plots. Experimental N addition in the field and
43 the laboratory confirmed that fertilized plants had a negative effect on grasshopper individual
44 performance and population size. The observed indirect effect of large vertebrate herbivores

on insect herbivores, mediated by plant quality, appears to be an important mechanism explaining interactions between distantly related herbivores. Our results suggest that plant nutrients might act as a cryptic mechanism determining interspecific relationships between herbivores that might help explain species coexistence and the maintenance of biodiversity.

Key words: *plant quality; indirect interactions; large herbivores; grasshoppers; nutrient requirement; species coexistence; body size; evolution; compensatory regrowth.*

INTRODUCTION

In natural ecosystems, trophic levels are commonly made up of a complex suite of species, often competing for food resources and space (Strong et al. 1984, Stam et al. 2014, Kartzinel et al. 2015). Competition theory predicts that ecological similarity among species enhances the intensity of their interactions (Schoener 1974, Connell 1983). Thus, in the case of interactions within the herbivorous guild, the vast majority of work has traditionally focused on closely related species of similar size (e.g., Denno et al. 2000, Odadi et al. 2011, Karban et al. 2012). However, competition among herbivores does not always conform to traditional paradigms and phylogenetic similarity can be a poor predictor of interaction strength in some cases (Hochberg and Lawton 1990, Gurevitch et al. 1992, Kaplan and Denno 2007, Eubanks and Finke 2014). Increasingly, ecologists are recognizing the fact that interactions among distantly related herbivores are prevalent and relevant, with far-reaching effects on community assembly, organization and dynamics (Belovsky 1986, Pringle et al. 2007, Bakker et al. 2013).

Large mammalian herbivores sharing host plants with smaller herbivores, such as arthropods, are a classic example of interactions among phylogenetically distinct groups. Recent meta-analytical reviews of the literature find that on average the effects of large herbivores on smaller animal taxa are negative (Foster et al. 2014, Takagi and Miyashita 2014, van Klink et al. 2015); however, there is considerable variation in the outcome of individual studies including positive (Cao et al. 2015), negative (Pringle et al. 2007), and neutral effects (Steen et al. 2005). While some of this context-dependency can be attributed to differences in the spatial and temporal scale of the study, differences across systems in the processes linking large and small herbivores also play a significant role (Takagi and Miyashita 2014).

The mechanisms underlying the effects of large herbivores on smaller herbivores include both direct and indirect pathways. Direct interactions result from incidental ingestion or trampling of smaller herbivores by large herbivores (Gómez and González-Megías 2002). Density-mediated indirect effects arise when large herbivores reduce plant biomass (Huntzinger et al. 2008, Vandegehuchte et al. 2017), thus limiting the availability of resources for other herbivores. Small herbivores typically lose out, since the outcome of competition between differently-sized herbivores tends to be highly asymmetrical (i.e. negative for the smaller herbivore and neutral for the larger, Gómez and González-Megías 2002). However, in situations where the smaller herbivore prefers a different host plant than that consumed by the large herbivore, facilitation (positive direct effects, i.e. commensalism/mutualism) may occur. As an example, Zhong et al. (2014) found that domestic sheep benefit grasshoppers by selectively consuming *Artemisia* forbs. The reduction in forb biomass enhances the ability of

grasshoppers to locate their preferred *Leymus* grass host plant. Trait-mediated indirect effects (or trait-mediated indirect interactions) result from herbivore-induced changes in host plant quality or architecture (e.g., Ohgushi 2005). For example, in the African savanna large grazers benefit other grazers either by stimulating re-growth of plants, which is usually of better quality than mature leaves, or by returning nutrients in the form of faeces and urine (McNaughton 1979, 1983). Feeding and trampling may also decrease the height and structural complexity of vegetation, thus altering the physical environment encountered by smaller herbivores (Huntzinger et al. 2008, Pringle 2007, Pagès et al. 2012). Simplification of plant structure may expose herbivores to harsh abiotic conditions or render them more vulnerable to predators (Suominen et al. 1999, van Klink et al. 2015), a type of indirect effect known as environment-mediated interaction modification (Wootton 1992). As highlighted above, large and small herbivores might interact through a diversity of pathways, which depend on the traits of the entire suite of herbivores, the traits of the plant community and environmental factors, making the prediction of the direction and strength of interactions a complex matter. For arthropods, in particular, responses are most often attributed to altered plant biomass and structure rather than changes in plant quality (Kruess and Tschardt 2002, Takagi and Miyashita 2014, Lind et al. 2017), but the impacts of plant quality are often not investigated directly (Moran 2014, Lind et al. 2017).

Indeed, alteration of plant quality is likely to have profound impacts on small herbivores, especially for some specialist insects (Belovsky 1997, Behmer and Joern 2008). In terrestrial systems, current paradigms usually assume that insect herbivores are nitrogen (N) limited due

to the mismatch between plant and insect tissues N content (Mattson 1980). Therefore, an increase in plant N content can result in higher herbivore performance. However, studies with grasshoppers (Clissold et al. 2006, Cease et al. 2012) and butterflies (Fischer and Fiedler 2000, Tao et al. 2014) have demonstrated that ingesting excessive N can lead to negative effects. Given the uncertainty in the response of insect herbivores to N, a direct test of the effects of large mammalian herbivory on plant quality and its consequences for phytophagous insects is needed.

Here, we explore the interaction between large domestic herbivores and small herbivorous insects in a semiarid grassland ecosystem. The dominant plant community in this system is the grass *Leymus chinensis*, with a variety of less abundant forb species (Zhu et al. 2012, Wang et al. 2017). The herbivore community is dominated by the large vertebrate *Bos taurus* (i.e. domestic cattle) and the small insect grasshopper *Euchorthippus unicolor*. Both cattle and *E. unicolor* grasshoppers prefer *L. chinensis* grass, and rarely feed on forbs (Zhong et al. 2014, Liu et al. 2015). Previous studies have found negative effects of ungulate grazing on grass-feeding grasshoppers due to reductions in plant biomass (Capinera and Sechrist 2012) and positive effects of ungulate grazing due to increased structural heterogeneity (Joern 2004), but no studies have systematically examined multiple mechanistic pathways, including altered plant quality. We used a set of field and laboratory manipulative experiments to investigate the potential pathways whereby large domestic herbivores and *E. unicolor* grasshoppers interact. We addressed the questions: (i) does large herbivore grazing affect the individual performance and population abundance of co-occurring *E. unicolor* grasshoppers,

and if so, what is the underlying mechanism? Specifically, we ask (ii) does large herbivore grazing modify plant resource availability (i.e. density-mediated indirect effect)?, (iii) does large herbivore grazing modify the microclimatic conditions experienced by small herbivores (i.e. environmentally-mediated indirect interaction)?, and (iv) does large herbivore grazing modify plant resource quality (i.e. trait-mediated indirect effect)?

MATERIALS AND METHODS

Study system and background

Our study was conducted at the Grassland Ecological Research Station, Northeast Normal University, Jilin Province, China (44°45' N, 123°45' E). The study site is located in the eastern region of the Eurasian steppe, where the climate is semiarid, with cold, arid winters and hot, rainy monsoonal summers. Annual mean temperature ranges from 4.6 to 6.4 °C, and annual precipitation varies between 280-400 mm, with 70% falling during the growing season, especially between June and August.

The meadow steppe in the area is dominated by the perennial rhizomatous grass *Leymus chinensis*, which has strong compensatory regrowth after grazing or clipping disturbance (Gao et al. 2008, Wang et al. 2017). The grass *L. chinensis* provides food and substrate to a great number and variety of native and domestic herbivores. Other common species at this site include grasses such as *Phragmites australis*, *Calamagrostis epigejos* and *Setaria viridis*; and forbs such as *Artemisia scoparia* and *Messerschmidia sibirica* (Liu et al. 2015). From the 1970s to 2011, this area was fenced to exclude livestock and human residents for grassland

management and conservation purposes.

The main large vertebrate herbivores in the area are domestic cattle (*Bos taurus*), which prefer the dominant grass *L. chinensis* and rarely feed on forb species (Liu et al. 2015, also see Appendix S1). The major invertebrate herbivores at the site are grasshoppers (Oedipodinae, Acrididae) and planthoppers (Homoptera, Cicadellidae) (Zhu et al. 2012). The grasshopper *Euchorthippus unicolor* dominates the herbivorous insect community, accounting for > 65% of all insect abundance annually (Zhong et al. 2014). *E. unicolor* usually displays one generation a year and displays five nymphal stages: eggs typically hatch in late July to early August, becoming adults by mid-August, and reaching peak densities by the end of August (Zhong et al. 2014). *E. unicolor* shares similar dietary preferences with domestic cattle: it feeds predominately on the dominant grass *L. chinensis*, while it seldom feeds on forb species (Zhong et al. 2014, Appendix S1). Thus, the diets of both dominant vertebrate and invertebrate herbivorous species in this semiarid grassland ecosystem are completely overlapping.

Domestic cattle were introduced in our study site in 2012, and their grazing is maintained at a light to moderate intensity (0.1-0.3 animal units/ha). Before the onset of cattle grazing in May 2012, five 50 × 50 m fenced (1.5 m high) cattle exclosures (ungrazed plots) were randomly established across the study site at 50 m intervals. Additional unfenced 50 × 50 m plots served as controls (grazed plots) in areas adjacent to each of the five ungrazed plots, about 2 m apart. Control grazed plots were used to monitor the responses of plant communities to cattle grazing.

Effects of cattle grazing on *E. unicolor* population density and performance

To investigate the effects of cattle grazing on *E. unicolor* density in grazed and ungrazed plots after 3 years of cattle grazing (question (i)), two parallel transects with a series of ten 0.25 m² rings were placed. Rings were left undisturbed for at least one day before grasshopper surveys. Densities were estimated by counting the number of *E. unicolor* grasshoppers in each ring (Joern 2004). Surveys were conducted on the 10th, 21st, 30th of August, and the 8th of September 2014. All surveys were conducted on sunny days with minimal cloud cover and no wind. We averaged grasshopper density for the 10 sampling rings to yield a single value for each plot (n = 5) on each of the 4 sampling days.

To assess the effects of cattle grazing on *E. unicolor* performance we determined survival rates, body size and mass gain in the grazed and ungrazed plots. In August 2014, we installed 2 circular cages (0.75 m² basal area × 1.1 m high) in each plot (20 cages in total). Each circular cage was constructed of iron rod frames, covered with 2-mm plastic mesh screen, and sunk 10 cm into the ground. Just before the deployment of the circular cages, the area was cleared of all aboveground invertebrates and predators using a vacuum cleaner. We then collected fourth-instar grasshoppers using sweep-nets on the adjacent grassland area. Before the nymphs were introduced into the cages, grasshoppers were separated according to sex, and their initial body mass was determined by weighing seven individuals at a time in one vessel using an analytical scale (Top Instrument, Zhejiang, China). Grasshoppers were introduced into each circular cage in groups of 14 individuals (7 females and 7 males), which matched observed densities in the study site. Survival was estimated by counting the number

of grasshoppers per cage every three days, starting on the 8th of August and ending on the 28th of August. Adult body mass and size measurements were determined again for all the remaining females and males from each circular cage. Mass gain was calculated by subtracting the initial mass from the adult mass (Specht et al. 2008). We measured grasshopper body length and femur length using an Electronic Digital Caliper (Guanglu Inc., Guilin, China). The average for the two circular cages within each plot was used in statistical analyses.

Effects of cattle grazing on vegetation abundance and microclimatic conditions

In order to test question (ii), i.e. whether cattle grazing influenced vegetation abundance, we examined the differences in plant communities in grazed and ungrazed plots. In August 2014, we established two 50 m parallel transects within each plot and measured plant community characteristics in ten 0.5 × 0.5 m quadrats along each transect, at about 4 m intervals. Within each quadrat, we identified plant species, counted the number of stems (to obtain plant density), visually estimated the percentage cover of all plant groups, and measured plant height on 5 individual stems for each plant group (to the nearest centimetre) using a ruler. We estimated the volume (in m³) of three different plant groups (*L. chinensis*, other grasses and forbs) by multiplying the mean area covered with the mean height of each of the plant groups in twenty quadrats in each plot (Huntzinger et al. 2008). We measured aboveground biomass by clipping standing plant material to 1 cm above ground level from 8 randomly located 0.5 × 0.5 m quadrats within each plot. For each plant variable, the values were averaged to yield a single value for each plot (the level of replication for this field

213 experiment, i.e. n=5).

214 In order to test question (iii), i.e. whether cattle grazing influenced within-canopy
215 microclimatic conditions, we evaluated light, temperature and humidity at 5 cm and 35 cm
216 above ground (grasshopper active zones) in August 2014, within the same quadrats used for
217 vegetation variable sampling. Photosynthetically active radiation (PAR) was measured on a
218 clear day with a GLZ-C-G PAR (photosynthetically active radiation) point sensor (Top
219 Instrument, Zhejiang, China). Ambient air temperature and relative humidity were measured
220 using an AR-847 digital thermo-hygrometer (Jinzhan Inc., Shenzhen, China).

221 ***Effects of cattle grazing on plant quality***

222 To test question (iv), i.e. whether large grazers influenced plant quality, the chemical and
223 physical traits of the dominant grass *L. chinensis* were measured in grazed and ungrazed plots.
224 We collected *L. chinensis* leaves from 8 randomly located 0.5 × 0.5 m quadrats within each
225 plot, in mid-August in 2014. Leaves (sheaths and stems were removed) were fresh- and
226 dry-weighed to determine water content. Then, dried samples were ground using a ball-mill
227 and analysed for nitrogen (N) and carbon (C) contents, and C:N ratio using a Vario MIRCO
228 cube Elemental Analyzer (Elementar GmbH., Hanau, Germany). Leaf toughness of *L.*
229 *chinensis* was measured on 10 individual leaves in each plot using an improved SY-S03
230 penetrometer (Shiya Inc., Shijiazhuang, China). All values were averaged to yield a single
231 value for each plot (resulting in 5 replicates, n=5).

232 ***Effects of nitrogen addition on *E. unicolor* grasshopper and *L. chinensis* grass***

233 Our results in 2014 suggested that cattle influenced *E. unicolor* grasshoppers by altering

the quality (plant N content) rather than quantity of their shared food resource (see Fig. 1-4 in the Results section below). Therefore, in 2015, we conducted a set of field and laboratory fertilisation experiments to investigate how increased N content in plants affected *E. unicolor* grasshoppers.

In April 2015, we set up eighteen 4×4 m plots that were randomly allocated to three levels of nitrogen enrichment (0, 10 and 17.5 g N/m^2 , $n = 6$). Nitrogen levels were chosen to produce an increase in plant N equivalent to the effects of grazing (10 g N/m^2) and even higher (17.5 g N/m^2). Nitrogen was applied as an aqueous solution of NH_4NO_3 ; with the control, unfertilised treatment receiving an equivalent volume of just water. Plots were fertilized in early May and again at the end of June.

In late August 2015, we measured the density of *E. unicolor* grasshoppers within each treatment field plot. We randomly placed one 1 m^2 quadrat in each of the eighteen plots. Each quadrat was carefully enclosed using a 1.5 m high shading screen barrier. All grasshoppers in each quadrat were then removed using a sweep-net and the number of *E. unicolor* grasshoppers was counted (Heidorn and Joern 1987). The survey was conducted only on sunny days with minimal cloud cover and no wind, with three replications. In early August 2015, we installed one cage into each of the treatment plots (18 cages in total). We then introduced 14 fourth-instar nymphs (7 females and 7 males) to each cage, and counted the survivors every three days for three weeks. We measured grasshopper survival rate, body size and mass gain in each cage using the methods described above.

In order to control for the potential confounding factors present in the field fertilisation

experiment, we simultaneously conducted a laboratory experiment to test the response of *E. unicolor* grasshoppers to plant N content. In early August 2015, we collected fourth-instar nymphs from the field and separated them according to sex. Then, we randomly assigned each nymph to one of three treatments containing plant material that had received 0, 10 or 17.5 g N/m². We stocked 10 individuals per sex per cage (in cages 0.08 m² basal area × 0.3 m high, 36 cages in total, n =6). Fresh *L. chinensis* grass was collected from each of the treatments from the field plots, offered in small water-filled plastic vials, and replaced every other day. Grasshoppers were monitored for three weeks, and then we measured grasshopper survival rate, body size and mass gain in each cage.

In mid-August 2015, we also collected *L. chinensis* leaves from each of the field treatment plots, and measured their C and N contents using the same methods previously described.

Statistical analyses

All data were tested for normality with the one-sample Kolmogorov-Smirnov test and homogeneity with Levene's test. Data for proportion surviving of *E. unicolor* grasshoppers in cages were arcsine square root transformed before analysis. Untransformed data are presented in the figures. Statistical analyses were performed using SPSS for Windows 19.0, unless otherwise noted.

For the large herbivore exclusion field experiment, we treated each plot as the experimental unit (n= 5 replicates). We examined the effect of cattle grazing on the abundance of *E. unicolor* grasshoppers over time by repeated measures analysis of variance

(PROC MIXED, SAS 9.4). We conducted paired t-tests to assess the effects of cattle grazing on the performance (survival, body size and mass gain) of male and female *E. unicolor* grasshoppers. Using the same method, we evaluated the effects of large herbivores on the total plant biomass for each plant functional group (*L. chinensis*, other grasses, and forbs), as well as for the physical and chemical leaf traits of *L. chinensis* grass (N content, C content, C:N ratio, toughness and water content of leaf). Finally, we again used a series of t-test to assess the effects of cattle grazing on microclimatic conditions in the plots (air temperature, air humidity and light penetration).

For both nitrogen addition experiments in the field and in the laboratory, we treated each cage as the experimental unit (n= 6 replicates each). We used one-way ANOVAs to determine the effects of nitrogen addition (0, 10 or 17.5 g N/m²) on grasshopper density and performance. We also performed one-way ANOVAs to assess the effects of N treatment on *L. chinensis* leaf N content. Tukey's multiple comparison was used as post hoc analysis of significant differences among factors.

RESULTS

Effects of cattle grazing on E. unicolor population density and performance

Cattle grazing had a negative effect on the abundance of *E. unicolor* grasshoppers ($F_{1,8}=19.47$, $P<0.01$; Fig. 1), and the effect of grazing was consistent over time ($F_{3,24}=1.35$, $P=0.28$). Grasshopper abundance increased in both treatments throughout the growing season before dropping off at the end of the season ($F_{3,24}=15.88$, $P<0.001$).

Female *E. unicolor* survival rates were 44% lower in grazed than in ungrazed plots (t_4

=2.960, $P=0.042$, Fig. 2a) and size (femur length) was reduced by 6% in grazed plots (t_4
=4.824, $P=0.008$; Fig. 2e), but mass gain was unaffected (Fig. 2c). Cattle grazing reduced
mass gain of male *E. unicolor* ($t_4=3.948$, $P=0.017$; Fig. 2d), but did not affect their survival
rate or femur length (Figs. 2b and 2f).

Effects of cattle grazing on vegetation and microclimatic conditions

Cattle grazing did not reduce plant biomass or volume significantly for any of the studied
plant functional groups, although negative trends were observed (Fig. 3, Appendix S2: Table
S1). Cattle grazing did not reduce plant cover or height significantly for any of the three plant
groups either (Appendix S2: Table S1).

Cattle grazing significantly increased nitrogen content of *L. chinensis* leaves by 38% (t_4
=8.997, $P=0.001$; Fig. 4a), but did not alter leaf carbon content (Fig. 4b). This resulted in a 27%
decrease in C:N ratio of *L. chinensis* leaves ($t_4=-7.372$, $P=0.002$; Fig. 4c). In contrast,
livestock grazing did not change any of the plant physical traits assessed, with *L. chinensis*
leaf toughness and water content remaining similar in grazed and ungrazed plots (Appendix
S2: Fig. S1).

Cattle grazing significantly increased air temperature by +0.77°C on average at 35 cm
above ground ($t_4=3.777$, $P=0.019$), but had no impacts on air temperature at 5 cm, air relative
humidity or PAR at any of the two levels above ground (Appendix S2: Table S2).

Effects of nitrogen addition on L. chinensis grass and E. unicolor grasshopper

Both fertilization treatments in the field (10 g N/m² and 17.5 g N/m²) significantly
enhanced the nitrogen content of *L. chinensis* leaves by 34 % and 122% compared to controls,

repectively ($F_{2,15}=2824.555$, $P<0.001$; Appendix S2: Fig. S4a), but did not affect leaf carbon content (Appendix S2: Fig. S4b). These changes led to 24% and 54% decreases in C:N ratios of *L. chinensis* leaves in 10 g N/m² and 17.5 g N/m² treatments compared to the control treatment ($F_{2,15}=706.497$, $P<0.001$; Appendix S2: Fig. S4c).

Nitrogen addition of 10 g N/m² and 17.5 g N/m² markedly decreased grasshopper density within each field plot by 44% and 51%, respectively ($F_{2,15}=4.180$, $P=0.036$; Appendix S2: Fig. S2). In addition, fertilization inputs significantly reduced *E. unicolor* performance. Specifically, survival rate and femur length of female *E. unicolor* grasshoppers were reduced in nitrogen-enriched plots (survival rate, $F_{2,15}=11.609$, $P=0.001$; femur length, $F_{2,15}=6.07$, $P=0.014$; Figs. 5a and 5e), while mass gain was unaffected (Fig. 5d). Similarly, femur length of male *E. unicolor* was also reduced in fertilised plots ($F_{2,15}=5.678$, $P=0.016$; Fig. 5f), while enrichment did not affect the survival rate or mass gain of male *E. unicolor* (Figs. 5b and 5d).

Consistent with results in the field fertilisation experiment, *E. unicolor* performance was also reduced when fed N-fertilised plant material in laboratory conditions. Survival rate and femur length of female *E. unicolor* grasshoppers were significantly lower when fed N-fertilized *L. chinensis* material (survival rate, $F_{2,15}=13.693$, $P=0.000$; femur length, $F_{2,15}=7.791$, $P=0.005$; see Appendix S2: Fig. S3a, e). Again, the survival rate and femur length of male *E. unicolor* were not affected by N enrichment, while mass gain of male *E. unicolor* was significantly reduced when fed fertilised plant material ($F_{2,15}=7.93$, $P=0.004$; Appendix S2: Fig. S3d).

DISCUSSION

Inter-specific interactions between closely related herbivorous species have been widely studied and typically result in negative effects for both species (i.e. competition), as a result of the exploitation of a limiting shared plant resource. As an example, competitive interactions have been found between wild ungulates and cattle (Odadi et al. 2011) or grasshoppers and caterpillars (Xi et al. 2013). However, the outcomes of interactions between distantly related herbivores with contrasting body sizes are complex and tend to be asymmetrical (Gómez and González-Megías 2002). This study demonstrates that large vertebrate herbivores (cattle) produce negative trait-mediated indirect effects on much smaller invertebrate herbivores (grasshoppers) in a grassland ecosystem, by modifying the quality (nitrogen content) rather than the quantity of shared plant resources. Counter intuitively, this effect was a result of the increased plant quality (higher N) in cattle grazed areas. Although high leaf nitrogen content is often thought to be beneficial for herbivores, our experiments show that nitrogen enrichment resulted in lower survival rates and reduced performance for the herbivorous grasshopper. When assessing interacting herbivores with contrasting body sizes, it is important to take into account not only the obvious competitive pathways resulting from exploiting similar limiting resources, but also the variety of complex indirect effects mediated by plant quality and/or microclimatic conditions.

We surmise that complex indirect effects are more likely to arise between pairs of differently-sized interacting herbivores than between similarly-sized herbivores. This is because apart from the competitive effects, resulting from exploiting a shared limiting

360 resource, large herbivores' grazing has the potential to modify canopy structure (Joern 2004),
361 which is the habitat of most small herbivores, and plant physico-chemical traits (Riipi et al.
362 2005), to which small herbivores, and especially insects, are known to be particularly
363 sensitive (Behmer 2009). Indeed, in our study, large herbivores did not change the quantity of
364 their shared plant resource, *L. chinensis*, but significantly increased the quality of this
365 dominant grass, which indirectly reduced grasshopper population density, survival and
366 performance.

367 Increased quality of *L. chinensis* tissues in cattle-grazed plots may be attributed to
368 compensatory regrowth and improved conditions for plant growth following grazing (i.e.
369 reduced intraspecific competition for light and increased nutrient inputs from foraging cattle
370 urine and faeces) (McNaughton 1979). Previous studies simulating grazing in a similar
371 system reported that *L. chinensis* is a highly grazing-tolerant species that can compensate or
372 even over-compensate moderate herbivory under adequate light and nutrient conditions (Gao
373 et al. 2008, Liu et al. 2012). In contrast to the non-significant change in *L. chinensis*
374 abundance, large herbivores significantly increased plant N content, thereby lowering leaf
375 C:N ratios. These results are consistent with earlier similar studies in boreal forests (Riipi et
376 al. 2005) and African savannas (McNaughton 1983) and alpine meadows (Cao et al. 2015). A
377 possible explanation for the observed leaf nitrogen increase in our grazed plots is that grazed
378 plants have been shown to increase chlorophyll and photosynthetic enzymes concentration in
379 remaining or new leaves, which consequently results in a higher total N concentration (Price
380 1991, Briske 1996).

381 Since plants in many terrestrial ecosystems are N-limited, it is often assumed that their
382 primary consumers would be as well (Mattson 1980). However, grazing-induced N
383 enrichment of *L. chinensis* leaves significantly reduced the individual performance, as well as
384 the population density of *E. unicolor* in our native grassland. Similarly, our nitrogen addition
385 experiment confirmed the patterns documented in the exclosure experiment: the negative
386 effect of large herbivores on *E. unicolor* was mediated by an increase of N content of the
387 common resource *L. chinensis*. While it could be argued that the field caging experiment did
388 not control for all possible confounding effects of N fertilization (e.g. shifts in plant species
389 composition, plant structure or microclimate), our results from the controlled laboratory study
390 were consistent with the results from the field, and confirmed the detrimental effects of
391 N-rich *L. chinensis* leaves on grasshopper performance. Several potential mechanisms can be
392 proposed to explain the negative effects of high plant N content observed on grasshopper
393 performance. Firstly, studies based on nutrient balance (i.e. geometric framework) document
394 that each organism has a specific dietary intake target for any given nutrient (Raubenheimer
395 and Simpson 1993). Hence, if the nutrient concentrations of an organism's overall intake fail
396 to meet the target required, the organism might end up with a surplus in one nutrient and a
397 deficit in other essential elements (Behmer and Joern 2008, Ibanez et al. 2017). In our study,
398 cattle grazing (as well as fertilisation experiments) significantly decreased C:N ratio of *L.*
399 *chinensis* leaves, roughly from a C:N = 40 to a C:N = 29 (and from C:N = 37 to C:N = 17 in
400 the fertilisation experiments), which probably produced an imbalance on *E. unicolor* nutrient
401 homeostasis, and thus resulted in a reduction of grasshopper performance. The latter

mechanism has been suggested to occur for other north Eurasian steppe grasshoppers adapted to low-nitrogen-high-carbohydrate diets (Cease et al. 2012). Secondly, nutrient-rich plants may reduce insect performance by affecting specific physiological processes such as increasing the metabolic costs of storing and excreting excess N (Boersma and Elser 2006). There is indirect evidence that such an approach does operate in aphids (Zehnder and Hunter 2009), however, because it is often difficult to directly measure the metabolic costs of excess N in insects, further detailed experiments are needed to clarify the plausibility of this mechanism. Finally, some physical and chemical plant traits could change concomitantly with high N content, which may be the actual drivers of the observed negative effects on herbivores (Vannette and Hunter 2011). However, in our system, the increased N content in leaves from grazed plots did not result in concomitant changes in toughness or water content of *L. chinensis*, suggesting that at least physical leaf traits were not responsible for the negative effects observed on *E. unicolor* insects. It is possible that N fertilization increased the concentration of N-based alkaloids in *L. chinensis* leaves. However, since grasses generally lack secondary metabolites, or if they do display deterrent compounds they tend to be C-based (i.e. lignin and tannins), the negative effects of N fertilisation on grasshoppers are unlikely to be related to secondary metabolites in *Leymus* grass (Tscharntke and Greiler 1995). What might be the evolved mechanism behind this phenomenon? We suggest that, the negative effects of nutrient rich resources on *E. unicolor* population density and performance is most likely a result of their long-term adaptation to an N-limited environment. Many fertilization experiments in the Eurasian steppe have documented that the dominant grass *L.*

chinensis is limited by nitrogen (Wang et al. 2017). Hence, *E. unicolor* grasshoppers might have specialised to exploit this nutrient-poor resource. Given that many grassland ecosystems are similarly low in N content, we surmise that the mechanisms observed in this study might be not an exception among insect herbivores feeding on low-N grasses. For example, many grasshopper species such as *Phoetaliotes nebrascensis* in North America (Joern and Behmer 1998), *Chortoicetes terminifera* in Australia (Clissold et al. 2006), and *Oedaleus asiaticus* in north Asia (Cease et al. 2012) have a preference for low quality grasses instead of other more nutrient-rich forbs and legumes, which might be a consequence of their adaptation to N-limited environments.

In terrestrial ecosystems, multiple herbivores share the same host plant, creating opportunities for a variety of direct and indirect effects to arise. Plant quality alteration as a response to herbivory conforms to the basis of trait-mediated indirect effects, which are pervasive in terrestrial systems (Ohgushi 2005). However, the effects of large vertebrate herbivory on plant quality are difficult to predict, and will depend on several extrinsic and intrinsic factors, including plant resistance strategy, plant life history and environmental conditions, as well as the intensity and frequency of herbivore attack (Briske 1996). Invertebrate herbivores, particularly specialist grasshoppers and caterpillars, usually reach maximal performance within a fairly limited range of plant nutrients (Behmer 2009). The optimal range of each nutrient for a given herbivorous species can be highly variable, which is most notable between different insect species (Behmer and Joern 2008), but it also occurs within a species as a result of developmental status, immediate physiological state and/or sex

(Behmer and Joern 1993, Hawlena and Schmitz 2010). Our results strongly suggest that large vertebrate herbivores (cattle) and small insect herbivores (*E. unicolor* grasshopper) instead of competing for a shared plant resource, actually interact indirectly through a trait-mediated interaction modification whereby cattle modify plant quality, which negatively influences grasshopper population densities, survival and performance. Understanding the precise mechanism of the negative effect of N-rich grass on this grasshopper merits further exploration and can provide new insights into trait-mediated indirect interactions between distantly related and differently sized herbivores.

Conclusions

Although large vertebrate herbivores are widely known to exert strong effects on invertebrate herbivores in terrestrial ecosystems, many questions surrounding these biotic interactions remain to be answered due to the paucity of carefully designed controlled experiments. Our study provides evidence for a mechanistic understanding of a trait-mediated indirect interaction, mediated by plant quality, that might be pervasive between differently-sized interacting herbivores. Uniquely, we show that when herbivores of contrasting sizes are involved, the outcomes of their interaction are driven more by the indirect effects of the large herbivore modifying plant traits, than by competition for a limiting shared plant resource. These less obvious but relevant pathways warrant more attention, given their potential generality. These findings further our understanding of the mechanisms for herbivore coexistence in natural ecosystems, and may have important implications for developing effective grazing management systems.

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FIGURE LEGENDS

FIGURE 1 Population density of grasshopper *Euchorthippus unicolor* in the grazed and ungrazed plots over 4 sampling dates in August and September 2014. Values are means \pm SE.

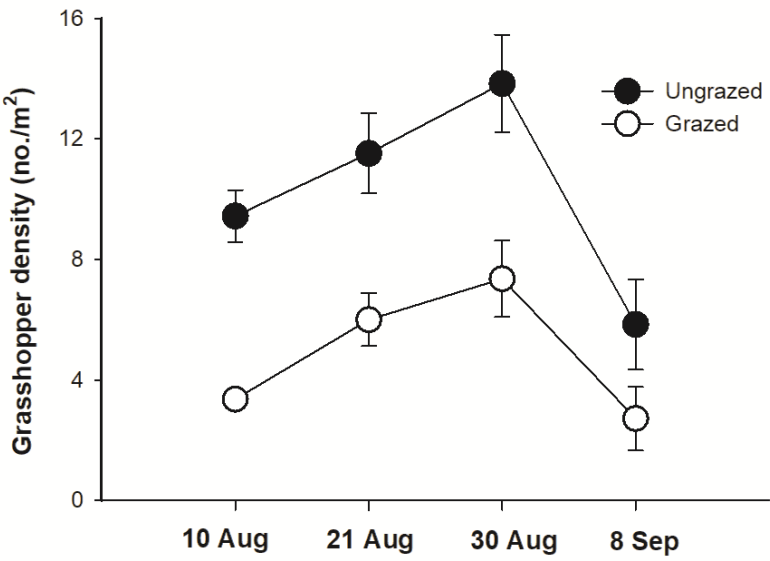
FIGURE 2 Performances of female and male grasshopper *Euchorthippus unicolor* in the grazed and ungrazed plots in August 2014: (a, b) survival rate, (c, d) mass gain, (e, f) body size. Seven female and seven male 4th stage nymphs were monitored in field circular cages installed on each plot for three weeks. Values are means \pm SE. The data are analyzed separately by sex, because female grasshoppers are significantly larger than males. Asterisk indicate significant difference (* = $P < 0.05$, ** = $P \leq 0.01$).

FIGURE 3 Biomass of (a) total, (b) *Leymus chinensis*, (c) other grasses, and (d) forbs in the grazed and ungrazed plots in August 2014. Values are means \pm SE.

FIGURE 4 Total (a) nitrogen content (percentage dry mass), (b) carbon content (percentage dry mass), and (c) C:N ratio of *Leymus chinensis* leaves in the grazed and ungrazed plots in August 2014. Asterisk indicate significant difference (** $P \leq 0.01$, *** $P \leq 0.001$). Values are means \pm SE.

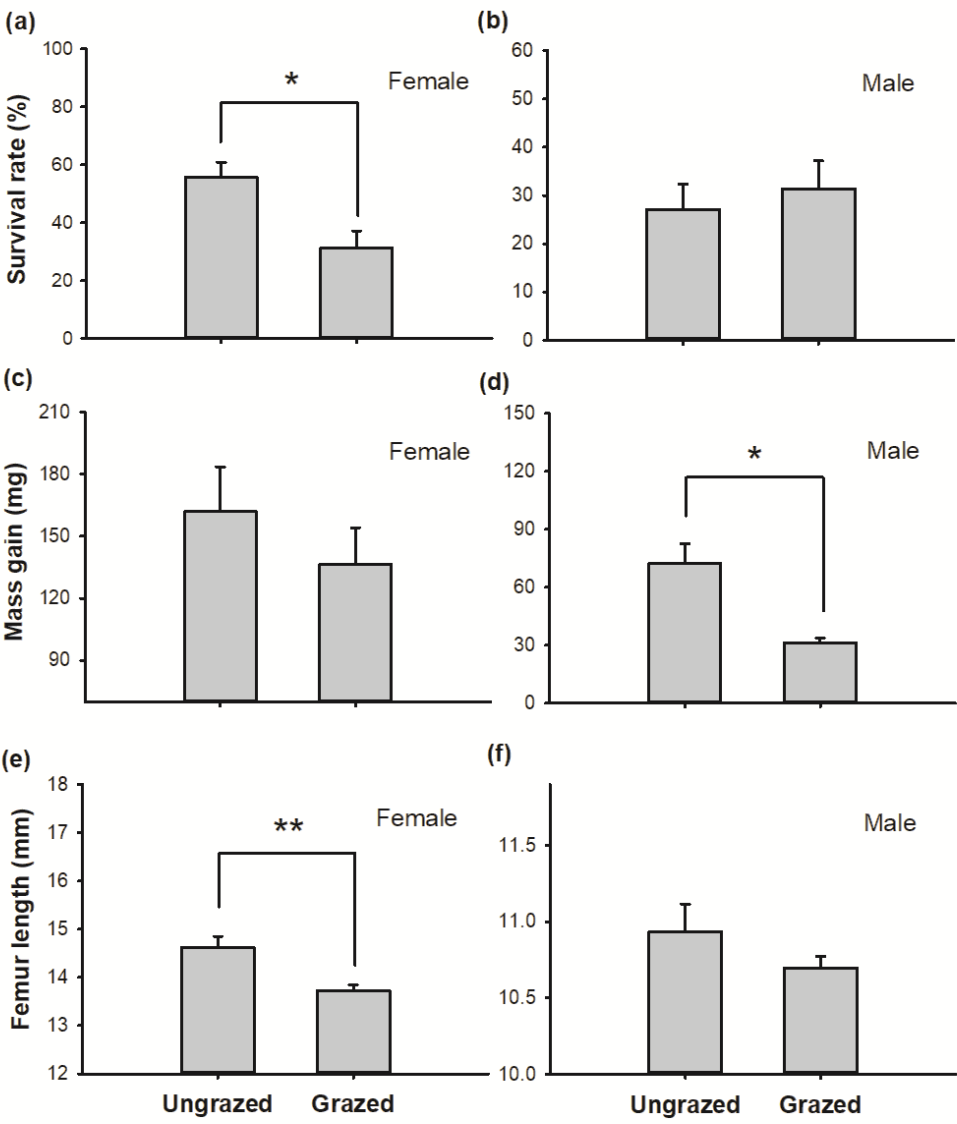
FIGURE 5 Effects of nitrogen application on grasshopper *Euchorthippus unicolor* performance in field circular cages. The (a, b) survival rate, (c, d) mass gain, (e, f) body size of female and male grasshopper of different nitrogen application treatments (0, 10 and 17.5 g N/m²). In August 2015, seven female and seven male 4th stage nymphs were monitored for three weeks. Different letters above the columns indicate significant differences ($P < 0.05$). Values are means \pm SE.

652 FIGURE 1

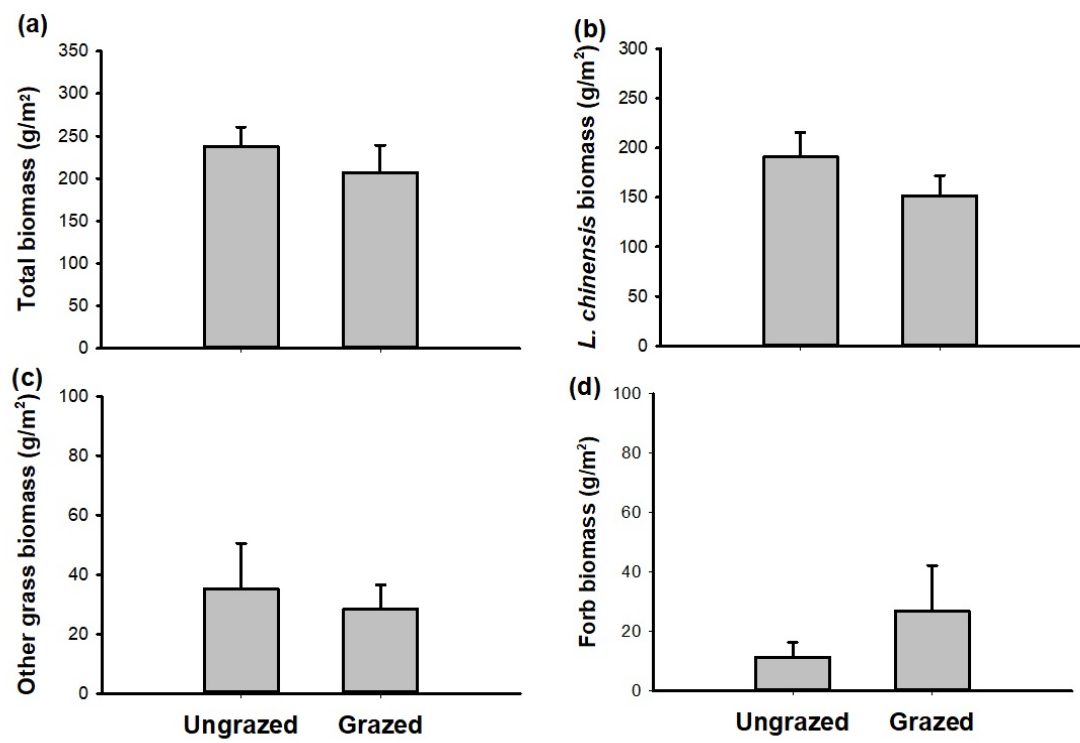


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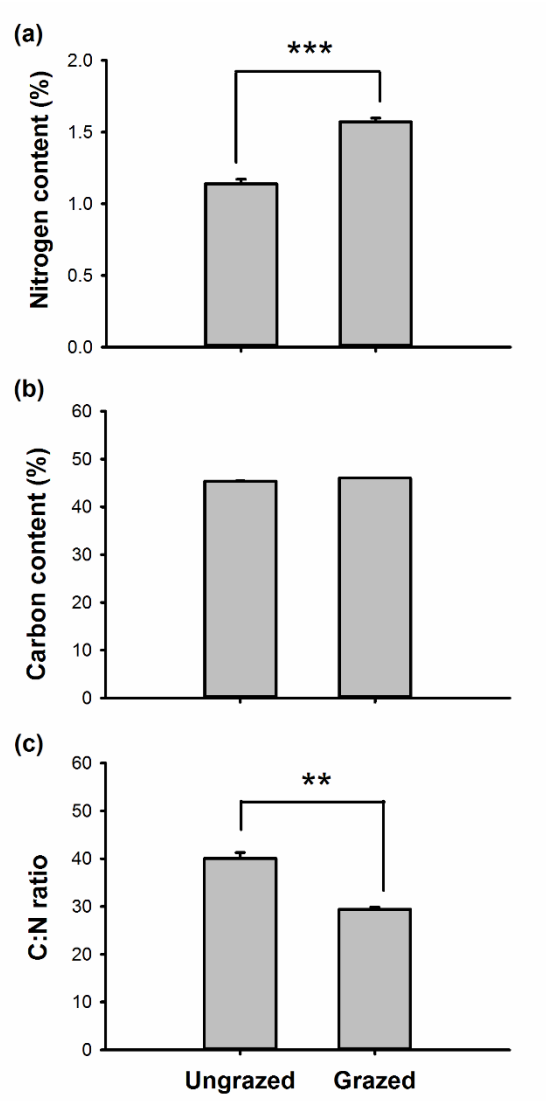
658 FIGURE 3



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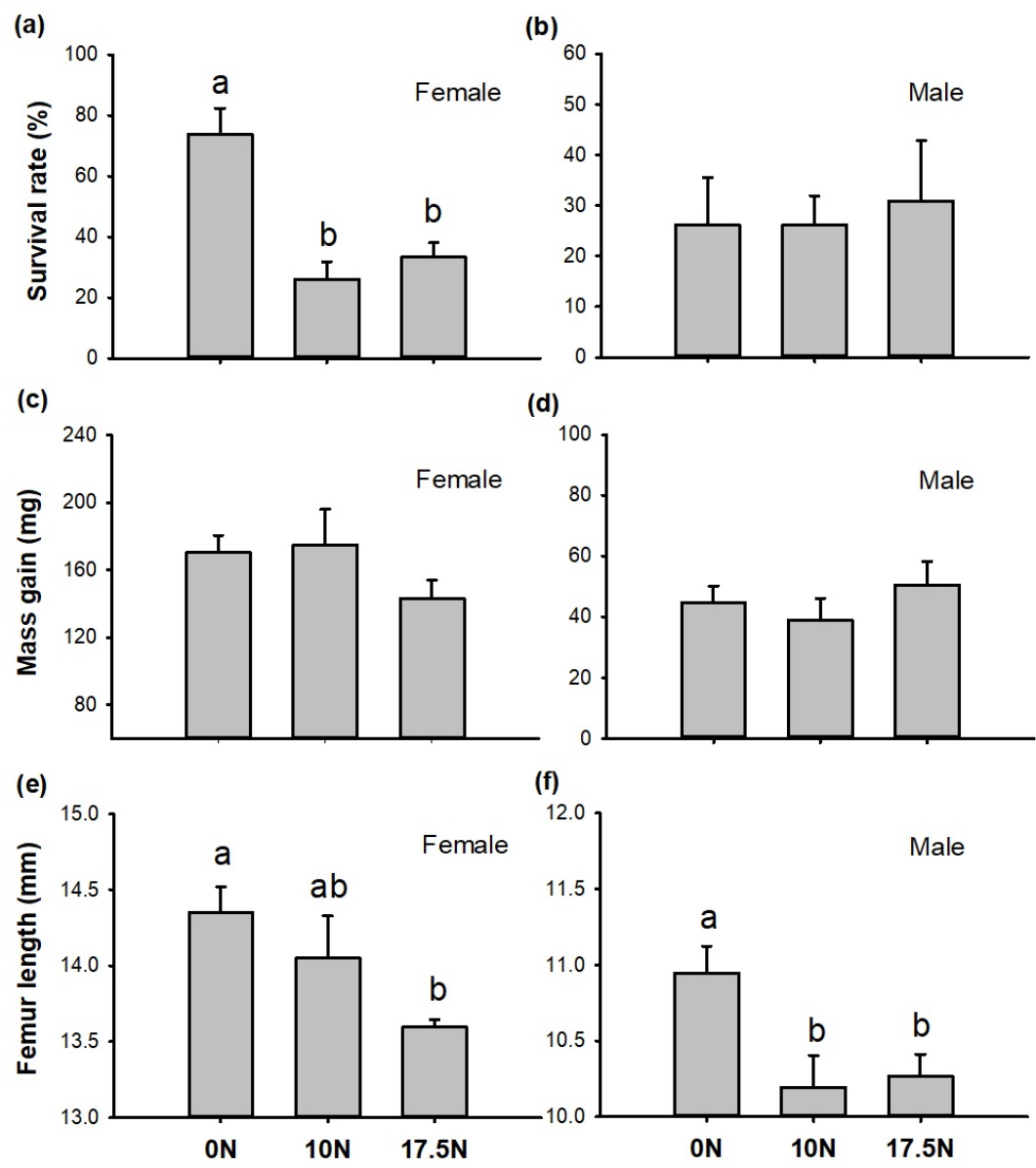
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661 FIGURE 4



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SUPPLEMENTAL MATERIAL

Appendix S1

A pre-experiment: Diet selection of large herbivores and grasshoppers

In late July 2014, we conducted a set of field and laboratory experiments to provide information about the dietary preferences of large herbivores and grasshoppers in our study system. We measured the grazing frequency of different plant groups (*Leymus chinensis*, other grasses, forbs) by large herbivores in the field. We established three parallel transects within each grazed plot, each transect consisted of 0.5×0.5 m quadrats at 2 m intervals. Where plants were grazed, the quadrat was assigned a value of one for that the plant group, otherwise 0. Then, values assigned for each plant group were summed, and divided by the number of quadrats to obtain a frequency of grazing use ranging from 0% to 100%. We observed feeding behavior of *Euchorthippus unicolor* grasshopper in the laboratory. In August 2013, we collected seven of the most common plant species and placed similar bundle-sizes of each plant species into small water-filled glass vials. We stocked 8 female individuals per cage and recorded the number of females feeding on the corresponding species every 40 minutes. The experiment started at 7: 00 am and finished at 4: 00 pm, with 10 replicates. Then, the numbers recorded for each plant species were summed, and sorted into three plant groups and divided by total number of grasshoppers to obtain a feeding frequency of grasshoppers.

For the diet selection of cattle and grasshoppers, we used a one-way ANOVA to evaluate the mean difference of large herbivore grazing frequency on each vegetation group in the

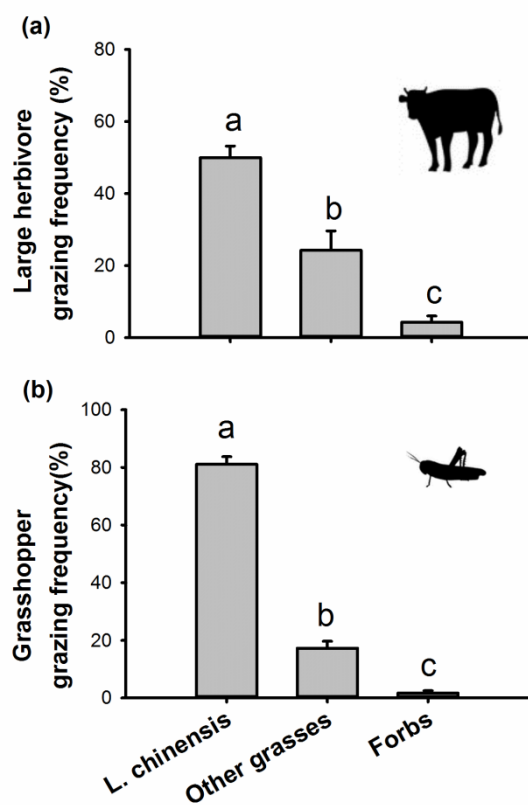
688 grazed plots and the mean difference of grasshoppers feeding frequency on each vegetation
689 group (including seven plant species) in a nine-hour lab observation. For the variables of
690 herbivore grazing frequency, Tukey's multiple comparison was used for post hoc analysis.

691

692 FIGURE S1 Diet preferences for large herbivores (cattle) and grasshoppers (*Euchorthippus*
693 *unicolor*) in eastern Eurasian steppe of China. (a) Grazing frequency of large herbivores on
694 each plant group in the grazed plots. (b) Feeding frequency of grasshoppers on three
695 vegetation groups (including seven species *Leymus chinensis*, *Phragmites australis*,
696 *Calamagrostis epigejos*, *Setaria viridis*, *Messerschmidia sibirica*, *Kalimeris integrifolia* and
697 *Artemisia scoparia*) in a nine-hour lab observation. Different letters above the columns
698 indicate significant differences ($P < 0.05$). Values are means \pm SE.

699

700 FIGURE S1



Diet selection experiments showed that large herbivores and grasshoppers had similar diet selection and both appeared to selectively consume the dominant grass *Leymus chinensis* (Appendix S1: Fig. S1).

Appendix S2

TABLE S1 Effects of large herbivores on plant community in August 2015. Relative aboveground density, cover, height and volume of total and the three plant groups (*Leymus chinensis*, other grasses and forbs) in the grazed and ungrazed plots. All data were tested with paired t-tests. Values are means \pm SE.

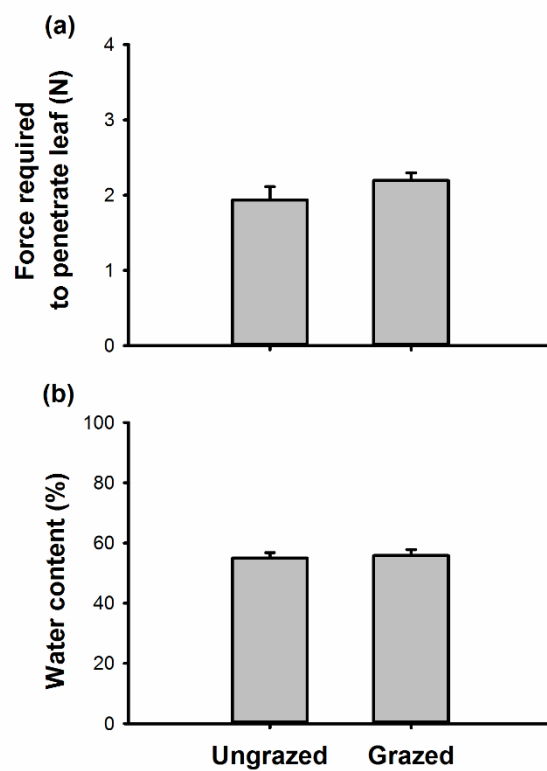
Plant community	Ungrazed	Grazed	<i>P</i>
Total			
Density (/m ²)	332.69 \pm 11.83	327.41 \pm 26.66	0.882
Cover (%)	85.20 \pm 4.16	81.06 \pm 3.55	0.558
Height (cm)	50.10 \pm 2.14	45.09 \pm 1.67	0.206
Volume (m ³)	0.11 \pm 0.008	0.09 \pm 0.001	0.083
<i>Leymus chinensis</i>			
Density (/m ²)	261.43 \pm 12.76	242.73 \pm 24.48	0.599
Cover (%)	57.14 \pm 2.87	50.30 \pm 3.48	0.332
Height (cm)	60.66 \pm 0.98	56.82 \pm 1.22	0.154
Volume (m ³)	0.09 \pm 0.005	0.07 \pm 0.005	0.194
Other grasses			
Density (/m ²)	46.97 \pm 5.57	61.94 \pm 15.08	0.493
Cover (%)	17.20 \pm 2.11	13.26 \pm 2.14	0.308
Height (cm)	58.67 \pm 2.45	48.25 \pm 4.28	0.113
Volume (m ³)	0.03 \pm 0.004	0.02 \pm 0.004	0.155
Forbs			
Density (/m ²)	24.29 \pm 7.88	22.74 \pm 5.46	0.840
Cover (%)	10.86 \pm 2.97	17.50 \pm 2.04	0.119
Height (cm)	30.96 \pm 3.49	30.20 \pm 1.97	0.839
Volume (m ³)	0.008 \pm 0.002	0.01 \pm 0.002	0.148

TABLE S2 Effects of large herbivores on microclimatic conditions at 5 cm and 35 cm above ground in August 2015. All data were tested with paired *t*-tests. Values are means \pm SE.

Microclimate	Ungrazed	Grazed	<i>P</i>
<i>5 cm ground height</i>			
Air relative humidity (%)	25.02 \pm 0.25	24.90 \pm 0.27	0.683
Air temperature (°C)	31.79 \pm 0.16	32.33 \pm 0.17	0.300
PAR (μ mol/m ² s)	243.29 \pm 24.27	330.80 \pm 58.85	0.273
<i>35 cm ground height</i>			
Air relative humidity (%)	23.60 \pm 0.23	23.25 \pm 0.14	0.345
Air temperature (°C)	31.90 \pm 0.16	32.67 \pm 0.15	0.019
PAR (μ mol/m ² s)	894.49 \pm 57.98	965.95 \pm 26.92	0.411

720 FIGURE S1 Effects of large herbivores on *Leymus chinensis* (a) toughness and (b) water
721 content in August 2014. Values are means \pm SE.

722 FIGURE S1



723

724

725

FIGURE S2. Effects of nitrogen application on grasshopper *Euchorthippus unicolor* density in field plots. We documented the number of grasshoppers in a 1 m² enclosure in the each 4 × 4 m nitrogen application treatment plot (0, 10 and 17.5 g N/m²) in August 2015. Different letters above the columns indicate significant differences ($P < 0.05$). Values are means \pm SE.

FIGURE S2

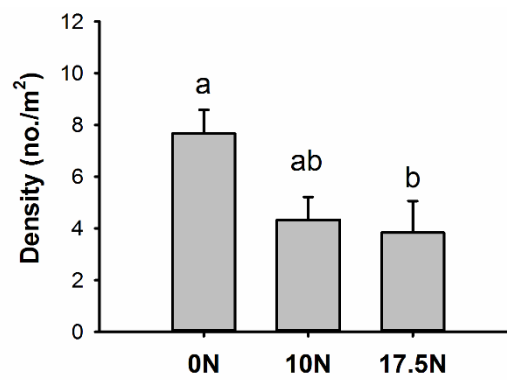


FIGURE S3 Effects of nitrogen application on grasshopper *Euchorthippus unicolor* performance in the lab. The (a, b) survival rate, (c, d) mass gain, (e, f) body size of female and male grasshopper reared on *Leymus chinensis* leaves from nitrogen application treatment plots: 0, 10 and 17.5 g N/m². In August 2015, ten female or ten male 4th stage nymphs were monitored for three weeks. Different letters above the columns indicate significant differences at $P < 0.05$. Values are means \pm SE.

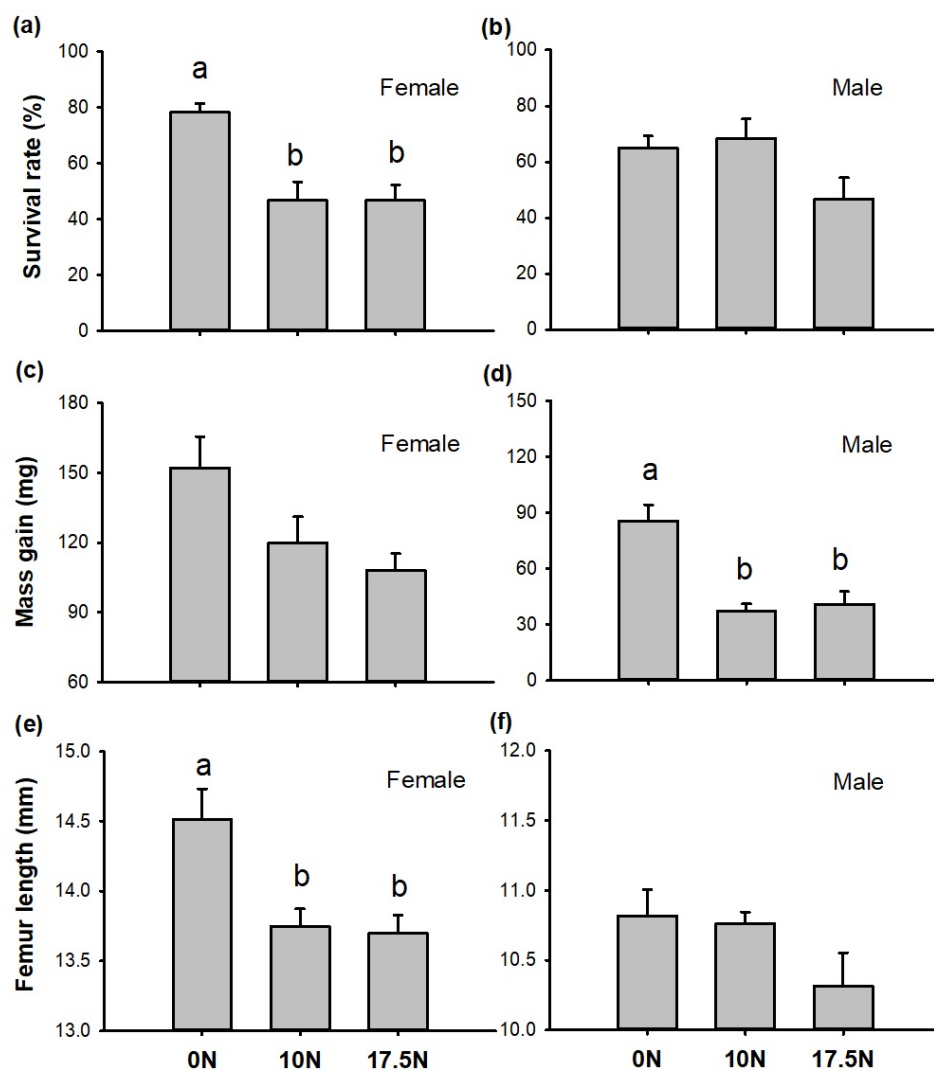


FIGURE S4 Leaf (a) nitrogen content (percentage dry mass), (b) carbon content (percentage dry mass), and (c) C:N ratio of *Leymus chinensis* from each nitrogen application treatment plot (0, 10 and 17.5 g N/m²) in August 2015. Different letters above the columns indicate significant differences ($P < 0.05$). Values are means \pm SE.

FIGURE S4

